Relationship between hepatic grayish-white solid nodules in horses imported from Canada and larval *Echinococcus multilocularis* infection

Tatsuro Hifumi, Tetsuya Tanaka, Emmanuel Pacia Hernandez, Kohei Akioka, Kaori Yamada, Yasutaka Imamura, Hitoshi Hatai, Noriaki Miyoshi

Abstract — Histopathological and genetic examinations were conducted on grayish-white solid hepatic nodules in 150 horses imported from Canada, in order to investigate larval *Echinococcus multilocularis* infection. Ten of the 150 horses (6.7%) were diagnosed with alveolar hydatid disease. The sequences of the mitochondrial cytochrome b genes obtained from all 10 polymerase chain reaction positive samples had 99 to 100% identity with the European haplotype E1 of *E. multilocularis.* Therefore, we concluded that the infections likely originated in Canada.

Résumé — Relation entre les nodules hépatiques solides blanc-grisâtre trouvés chez des chevaux importés du Canada et l'infection larvaire à *Echinococcus multilocularis*. Des examens histopathologiques et génétiques ont été effectués sur des nodules hépatiques solides blanc-grisâtre observés chez 150 chevaux importés du Canada afin d'étudier l'infection larvaire à *Echinococcus multilocularis*. Dix des 150 chevaux (6,7 %) ont reçu un diagnostic de maladie hydatique alvéolaire. Les séquences des gènes mitochondriaux du cytochrome b obtenus à partir des 10 échantillons positifs par réaction d'amplification en chaîne par la polymérase ont montré une identité de 99 à 100 % avec l'haplotype européen E1 d'*E. multilocularis.* L'haplotype d'*E. multilocularis* obtenu à partir de cette étude suggère que les infections sont probablement originaires du Canada.

(Traduit par Dr Serge Messier)

Can Vet J 2021;62:285–288

Alveolar hydatid disease, caused by infection with the larval stage of *Echinococcus multilocularis,* is a zoonosis of human health significance (1). *Echinococcus multilocularis* is widely distributed in the Northern Hemisphere, including extensive endemic regions in North America, Europe, and Asia (1,2). In North America, *E. multilocularis* was thought to be restricted to 2 regions: the northern tundra zone that begins on the west coast of Alaska and extends north and east to occupy most of the Canadian Arctic, and the north-central region that includes southern portions of Canada (Alberta, Saskatchewan, and Manitoba) and 13 contiguous states in the United States (3). However, recent reports suggest that the distribution is expanding; a dog that lived in British Columbia and had no history outside that province was diagnosed as having alveolar hydatid disease (4), and wild canids in that region have been diagnosed with *E. multilocularis* infection (5). In Canada, *E. multilocularis* maintains its life cycle between wild canids (e.g., foxes and coyotes) and dogs as definitive hosts and small rodents (e.g., voles) as intermediate hosts (6).

Horses, like humans and pigs, are dead-end hosts of *E. multilocularis* and become infected through oral ingestion of eggs in the feces of definitive hosts (7). Lesions resulting from larval *E. multilocularis* infection are most common in the liver (7). According to a previous report from Japan (7), 52.6% of slaughtered racehorses with hepatic grayish-white solid nodules had infections with larval *E. multilocularis.* These horses may have been infected by larval *E. multilocularis* in Hokkaido, northern Japan, where most racehorses are born, and alveolar hydatid disease is endemic. Since 2004, Japan has imported more than 2500 live horses from Canada annually (8). Most of these horses are draft horses that are fattened on a farm in Kumamoto Prefecture, southern Japan, for 3 to 4 mo after being imported from Canada, and then slaughtered in Kumamoto Prefecture. An interview with veterinary meat inspectors from the Kumamoto Prefectural meat inspection office revealed that hepatic grayish-white solid nodules were detected at postmortem inspection in approximately 10% of all imported horses. The purpose of this study was to investigate the prevalence of larval *E. multilocularis* infection in slaughtered horses, imported from Canada, with hepatic grayish-white solid nodules.

From August 2017 to February 2018 and from August 2018 to January 2019, 2326 draft horses were brought to an abbatoir in Kumamoto Prefecture. According to interviews with

Transboundary Animal Diseases Research Center (Hifumi), Laboratory of Veterinary Histopathology (Hifumi, Hatai, Miyoshi), Laboratory of Infectious Diseases (Tanaka, Hernandez), Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan; Kumamoto Prefectural Meat Inspection Office, 1314 Sosaki, Shichijo-machi, Kikuchi, Kumamoto, 861-1344, Japan (Akioka, Yamada, Imamura).

Address all correspondence to Dr. Tatsuro Hifumi; e-mail: k3682515@kadai.jp

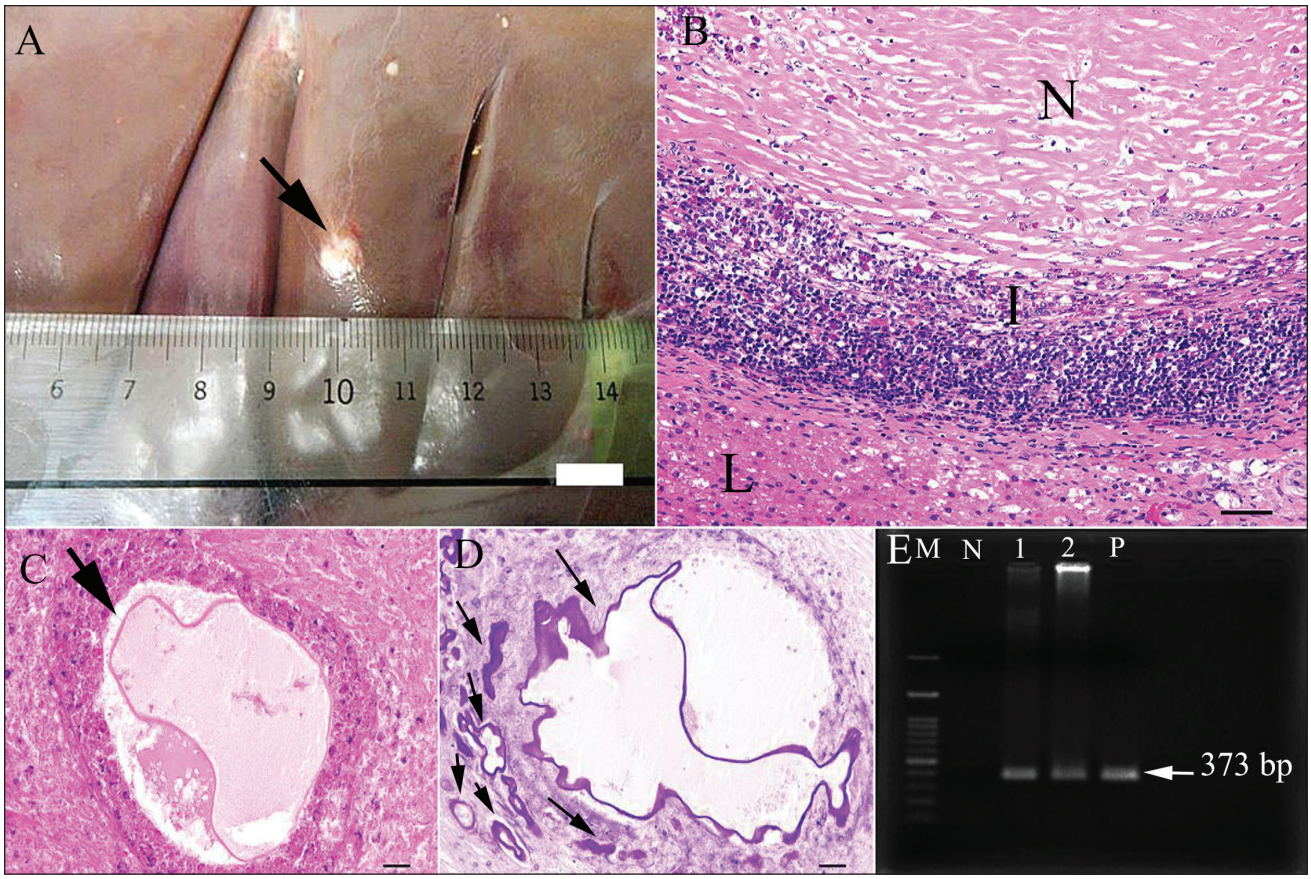

Figure 1. A – Hepatic grayish-white solid nodule 8 mm in diameter (arrow) (Case No. 3). Bar = 1 cm. B – The nodule (N) consisted of mature collagen fibers and well-demarcated with the liver parenchyma (L). Mild-to-moderate infiltration of inflammatory cells, consisting of lymphocytes, eosinophils, and macrophages (I), was observed at the periphery of the fibrous nodule (Case No. 3). Hematoxylin and eosin (H&E) stain. Bar = 50 μm. C – The laminated layer (arrow) was embedded in fibrous connective tissue (Case No. 3). H&E stain. Bar = 20 μm. D – Several laminated layers (arrows) were positive for Periodic acid–Schiff (PAS) stain (Case No. 3). PAS stain. Bar = 20 μm. E – Photograph of the cases that were positive for the mitochondrial 12S rRNA PCR. M – size marker; N – negative control (sterile distilled water); 1 – Case No. 3; 2 – Case No. 4; P – Hokkaido isolate of *E. multilocularis* (Nemuro strain) as a positive control.

the domestic livestock dealers who brought the horses to the abbatoir, these horses were imported to Japan at 2 to 16 y of age after being raised on a farm in Alberta, Canada. The horses did not show any clinical signs during the antemortem examination. During the postmortem examination, however, single or multiple hepatic grayish-white solid nodules were observed in 150 horses. The liver nodules ranged in size from 1 to 15 mm in maximum diameter. One nodule from each horse was collected and bisected. If there were multiple nodules in the liver of a horse, the biggest nodule was collected. After bisecting the nodule, 1 part was submitted for histopathological examination and the other part was frozen and stored at −20°C for genetic examination. For histopathological examination, the nodules were fixed in 10% neutral-buffered formalin, decalcified, and neutralized. Then, they were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) and Periodic acid–Schiff (PAS) stains. For genetic examination, 25 mg of tissue were collected from the nodular lesion and DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) was performed to amplify the mitochondrial 12S rRNA and cytochrome b *(cob)* genes of *E. multilocularis*, and the mitochondrial cytochrome c oxidase subunit 1 *(cox1)* gene of the genus *Echinococcus,* according to previous reports (9–11). Primers and conditions used were: i) for the 12S rRNA: forward (5′-TTAAGATATATGTGGTACAGGATTAGATACCC-3′) and reverse (5′-AACCGAGGGTGACGGGCGGTGTGTACC-3′) (9), with an initial denaturing at 94°C for 2 min, followed by 35 cycles at 98°C for 10 s, 58°C for 30 s, and 68°C for 30 s; ii) for the *cob* gene: forward (5′-TGCGTTATTGGCATATGG TAG-3′) and reverse (5′-GTGCCACCCTCAGTTACT-3′) (10), with an initial denaturing at 95°C for 3 min, followed by 40 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 5 min; and iii) for the *cox1* gene: forward (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and reverse (5′-TAAAGAAAGAACATAATGAAAATG-3′) (11), with an initial denaturing at 95°C for 5 min, followed by 35 cycles at 94°C for 50 s, 45°C for 50 s, and 72°C for 50 s, and a final extension at 72°C for 10 min. The PCRs for the 12S rRNA, *cob* and *cox1* genes yielded bands of 373, 693, and 446 bp, respectively. Amplification reactions were performed in a total volume of 12.5 μL, containing 1.0 μL of DNA template,

Table 1. Data for 10 horses infected with larval *Echinococcus multilocularis* imported from Canada and slaughtered in Japan.

Case number	Sex	Age[a] (y)	Nodule size[b] (mm)	Histopathology (laminated layer)	12S rRNA PCR
1	F	5	8	−	+
2	CM	2	2	−	+
3	CM	7	8	+	+
4	CM	2	6	+	+
5	CM	4	4	−	+
6	CM	3	10	−	+
7	F	8	4	−	+
8	CM	3	13	+	+
9	CM	3	1	−	+
10	F	6	7	+	+

F — Female; CM — Castrated male.
[a] The information on the age was obtained from an application form for slaughter inspection.
[b] Maximum diameter.

6.25 μL of 2× Gflex PCR Buffer (Takara Bio, Shiga, Japan), 0.15 μL of each primer (10 pM), 0.15 μL of Tks Gflex DNA polymerase (1.25 U/μL; Takara Bio), and 4.8 μL of sterile distilled water. A direct sequencing method using a 3730 × l DNA Analyzer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was performed on the PCR products of positive samples to determine the nucleotide sequence. The sequences that were obtained were compared to registered sequences in the National Center for Biotechnology Information nucleotide database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Cases that had laminated layers identified by histopathology and were positive by PCR, or cases that did not have the laminated layers by histopathology and were positive by PCR, were diagnosed as having alveolar hydatid disease.

A liver nodule in a slaughtered horse is shown in Figure 1A. Grossly, the nodule was solid, well-circumscribed and grayish-white. Histopathologically, the nodules collected from all 150 horses were a fibrous nodule consisting of mature collagen fibers accompanied by minimal necrosis and mild to moderate infiltration of eosinophils, lymphocytes, and macrophages at the periphery of the nodule (Figure 1B). In 14 of the 150 horses, there were laminated layers embedded in the fibrous connective tissue (Figure 1C) and the samples were positive for PAS stain (Figure 1D). Brood capsules or protoscolex formation was not observed in any case. Based on the genetic examination, 10 of 150 horses were positive by the 12S rRNA PCR, including 4 horses with laminated layers in lesions and 6 horses that lacked laminated layers. However, 10 horses that had the laminated layers histopathologically were negative for the 12S rRNA and *cox1* genes by PCR. Positive results for the 12S rRNA PCR are shown (Figure 1E). The 12S rRNA sequences obtained from all 10 PCR positive samples had 100% identity to *E. multilocularis* (GenBank accession numbers EU043372 and L49455). The *cob* sequences obtained from all 10 PCR positive samples had 99 to 100% identity with the European haplotype E1 of *E. multilocularis* isolated from Austria (GenBank accession number AB461395). Based on these results, 10 horses, including 4 horses with laminated layers histopathologically that were positive for PCR and 6 horses without the laminated layers histopathologically that were positive for PCR, were diagnosed as having alveolar hydatid disease. Thus, the prevalence in horses with hepatic grayish-white solid nodules was 6.7% (10/150). Sex and age of the 10 horses infected with larval *E. multilocularis,* size of the liver nodules, and the results of histopathological and genetic examinations are shown (Table 1). The infected horses included 7 castrated males and 3 females, ranging from 2 to 8 y of age. Liver nodules ranged in size from 1 to 13 mm in maximum diameter.

Based on histopathological and genetic examinations, we demonstrated that some horses imported from Canada were infected with larval *E. multilocularis.* Some cases in the present study had discrepant histopathological and genetic results. In cases with the laminated layers in a lesion that was negative by the 12S rRNA PCR, cystic echinococcosis due to larval *Ecchinococcus equinus* should be considered as a differential diagnosis (4,12). However, these cases were negative with the *cox1* PCR identifying the genus *Echinococcus* in the present study. In cases that had the laminated layers histopathologically and were negative for the 12S rRNA and *cox1* genes by PCR, a definitive diagnosis could not be made. The reason for the low sensitivity of the PCR in these cases remains unknown, but a more sensitive technique such as quantitative real-time PCR may be needed for further confirmation. The PCR-positive cases that lacked the characteristic laminated layers indicated that the mitochondrial 12S rRNA gene of *E. multilocularis* was present, even if the parasites were not detected histopathologically. We concluded that PCR would be a useful approach for an epidemiological survey of *E. multilocularis* because the diagnosis can be made even if the laminated layer in the lesion is not detected histopathologically.

The horses infected with larval *E. multilocularis* in the present study were imported from Canada, fattened and slaughtered in Kumamoto Prefecture, southern Japan. According to a previous report from Japan (13), from 1999 to 2018, *E. multilocularis* from humans and dogs was detected mainly in Hokkaido, northern Japan where alveolar hydatid disease is endemic, and not in Kumamoto Prefecture. Conversely, before being imported to Japan, the horses infected with larval *E. multilocularis* in the present study had been raised in Alberta, where alveolar hydatid disease is endemic (3). Perhaps the area in which these horses were raised in Canada has become contaminated with *E. multilocularis* eggs in the feces of definitive hosts and the horses were infected in Canada. In support of this hypothesis, the European-type strain (haplotype E1) of *E. multilocularis* was isolated from horses imported from Canada in the present study. In a previous report from Japan (14), the Asian-type strains (haplotypes A3 and A4) were detected in a fox and a vole. A recent study in Alberta, Canada reported that the European-type E1 strain was detected in coyotes (15). Considering these facts, our identification of the haplotypes of *E. multilocularis* suggests that the infections likely originated in Canada rather than Japan. This is the first report concerning strain-typing of *E. multilocularis* isolated from horses.

In conclusion, some of the horses imported from Canada and slaughtered in Japan which had grayish-white solid hepatic nodules were infected with larval *E. multilocularis.* The *cob* sequences obtained from PCR positive samples had 99 to 100%

identity with the European-type E1 strain of *E. multilocularis,* suggesting the infections likely originated in Canada rather than Japan.

Acknowledgment

We are grateful to Dr. Hirokazu Kouguchi of the Department of Infectious Disease, Hokkaido Institute of Public Health, for providing the positive samples for PCR. CVJ

References

1. Deplazes P, Eckert J. Veterinary aspects of alveolar echinococcosis — A zoonosis of public health significance. Vet Parasitol 2001;98:65–87.
2. Moro P, Schantz PM. Echinococcosis: A review. Int J Infect Dis 2009; 13:125–133.
3. Massolo A, Liccioli S, Budke C, Klein C. *Echinococcus multilocularis* in North America: The great unknown. Parasite 2014;21:73.
4. Peregrine AS, Jenkins EJ, Barnes B, et al. Alveolar hydatid disease *(Echinococcus multilocularis)* in the liver of a Canadian dog in British Columbia, a newly endemic region. Can Vet J 2012;53:870–874.
5. Gesy K, Hill JE, Schwantje H, Liccioli S, Jenkins EJ. Establishment of a European-type strain of *Echinococcus multilocularis* in Canadian wildlife. Parasitology 2013;140:1133–1137.
6. Catalano S, Lejeune M, Liccioli S, et al. *Echinococcus multilocularis* in urban coyotes, Alberta, Canada. Emerg Infect Dis 2012;18:1625–1628.
7. Goto Y, Sato K, Yahagi K, et al. Frequent isolation of *Echinococcus multilocularis* from the livers of racehorses slaughtered in Yamagata, Japan. Jpn J Infect Dis 2010;63:449–451.
8. Uma Kankei Shiryou, 2016 (in Japanese) [homepage on the Internet]. Ministry of Agriculture, Forestry and Fisheries 2017 [updated 2017 March]. Available from: https://www.maff.go.jp/j/chikusan/kikaku/lin/attach/pdf/sonota-25.pdf Last accessed January 21, 2021.
9. Dinkel A, von Nickisch-Rosenegk M, Bilger B, Merli M, Lucius R, Romig T. Detection of *Echinococcus multilocularis* in the definitive host: Coprodiagnosis by PCR as an alternative to necropsy. J Clin Microbiol 1998;36:1871–1876.
10. Gesy KM, Jenkins EJ. Introduced and native haplotypes of *Echinococcus multilocularis* in wildlife in Saskatchewan, Canada. J Wildl Dis 2015;51: 743–748.
11. Bowles J, Blair D, McManus DP. Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. Mol Biochem Parasitol 1992;54:165–173.
12. Simsek S, Roinioti E, Eroksuz H. First report of *Echinococcus equinus* in a donkey in Turkey. Korean J Parasitol 2015;53:731–735.
13. Echinococcosis in Japan, 1999–2018 [homepage on the Internet]. Infectious Agents Surveillance Report Vol. 40 p.33–34 [updated 2019 March]. Available from: https://www.niid.go.jp/niid/en/iasr-vol33-e/865-iasr/8695-469te.html Last accessed January 21, 2021.
14. Nakao M, Xiao N, Okamoto M, Yanagida T, Sako Y, Ito A. Geographic pattern of genetic variation in the fox tapeworm *Echinococcus multilocularis.* Parasitol Int 2009;58:384–389.
15. Gesy KM, Schurer JM, Massolo A, et al. Unexpected diversity of the cestode *Echinococcus multilocularis* in wildlife in Canada. Int J Parasitol Parasites Wildl 2014;3:81–87.